Effects of *Anemarrhena asphodeloides* on Focal Ischemic Brain Injury Induced by Middle Cerebral Artery Occlusion in Rats

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The preventive effect of *Anemarrhena asphodeloides* BUNGE (Liliaceae), a traditional Chinese medicine, on ischemia-reperfusion-induced brain injury was evaluated in the rat brain. Ischemia was induced by intraluminal occlusion of the right middle cerebral artery for 2 h and reperfusion was continued for 22 h. Water extract of *Anemarrhena asphodeloides* (WEAA) was orally administered promptly prior to and 2 h after reperfusion. Total infarct volume and edema in the ipsilateral hemispheres of ischemia-reperfusion rats were significantly reduced by treatment with WEAA in a dose-dependent manner (p<0.05). The therapeutic window of WEAA was 3 h in this ischemia-reperfusion rat model. WEAA also significantly inhibited increased neutrophil infiltration of ischemic brain tissue as estimated by myeloperoxidase (MPO) activity and immunohistochemical analysis. MPO-positive cells were markedly reduced by WEAA administration in striatal and cortical areas. These findings suggest that WEAA plays a crucial protective role in ischemia-induced brain injury, and suggest that WEAA could serve as a lead medicinal herb for the development of neuroprotective agents following transient focal ischemic brain injury.

Key words *Anemarrhena asphodeloides*; ischemia-reperfusion; neutrophil; myeloperoxidase (MPO)

Ischemic stroke is a leading cause of disability especially in elderly persons. The advancement of intravascular techniques and thrombolytic agents, especially the introduction of recombinant tissue plasminogen activator (rtPA), have reduced functional deficits within an optimal time window in stroke patients. The progression and extent of brain injury resulting from cerebral ischemia is related to several reperfusion mechanisms, many of which involve post-injury inflammatory response elements. These inflammatory elements include early neutrophil response, which occurs as soon as 4 h after reperfusion, and delayed macrophage infiltration that occurs several days later. Several lines of evidence have shown that neutrophils play an important role in the development of ischemic brain damage, and indicate that the depletion of circulating neutrophils or inhibition of neutrophil infiltration is thought to ameliorate cerebral ischemic injury.

*Anemarrhena asphodeloides* BUNGE (Liliaceae) is widely used in Chinese and Korean traditional medicines. The rhizoma of *A. asphodeloides* are known to have anti-diabetic, anti-platelet aggregation, and diuretic activities. Tinosaponins isolated from the rhizoma of *A. asphodeloides* have been shown to suppress platelet aggregation and both suppress and enhance superoxide generation in human neutrophils. These effects were dependent on the combination of tinosaponin species present and on the nature of the stimulus compound. However, no reports have been issued on the neuroprotective effects of *A. asphodeloides*.

In the present study, we investigated the effects of standardized water extract of *A. asphodeloides* (WEAA) on ischemic brain damage induced by focal ischemia-reperfusion in rats as well as its influence on neutrophil infiltration. Inflammatory cell infiltration was quantified by assaying myeloperoxidase (MPO) activity, which is mainly located in neutrophil primary granules. Immunohistochemical analysis was performed using anti-MPO antibody in the striatal and cortical areas. The antioxidant activity of WEAA was also assessed by determining the radical scavenging effect of the stable 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical.

MATERIALS AND METHODS

**Materials** 3-Methyl-1-phenyl-2-pyrazolium-5-one (MCI-186), 2,3,5-triphenyltetrazolium chloride (TTC), O-di-anisidine, hexadecyltrimethylammonium bromide (HTAB), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), and (-)-epicatechin were purchased from Sigma Chemical Co. (U.S.A.). Anti-MPO antibody was obtained from Santa Cruz Biotechnology, Inc. (U.S.A.) and secondary antibody and ABC kit from Vector (U.S.A.). Silicone (Xantopren) and hardener (Optosil-Xantopren Activator) were obtained from Bayer Dental (Germany). All other materials were of the highest grade commercially available.

**Animals** Male Sprague-Dawley rats weighing 260—280 g were purchased from Orient Co., Ltd. (a branch of Charles River Laboratories; Seoul, Korea). The rats were housed 4 or 5 per cage, allowed free access to water and food, and maintained under constant temperature (23±1 °C) and humidity (60±10%) under a 12-h light/dark cycle (light on 07.30—19.30 h). Animal treatment and maintenance were conducted in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and with the Animal Care and the Use Guidelines of Kyung Hee University, Korea.

**Preparation of Herbal Extracts** Dried rhizoma of *A. asphodeloides* were purchased at the Kyungdong Oriental drug store (Seoul, Korea) in 2003. The material obtained was authenticated by Professor Chang Soo Yook of the Depart-
ment of Oriental Pharmaceutical Science, College of Pharmacy, Kyung Hee University, Korea. The voucher specimens (No. KHOPS-03-017) have been deposited at the herbarium located in the College of Pharmacy, Kyung Hee University. Rhizoma were extracted with water for 2 h (80–100°C). The extract obtained was filtered and concentrated on a water bath under vacuum then frozen and lyophilized (Eyela, model FD-5N, Japan) to yield a water extract (defined as WEAA), which was stored at −20°C until required (yield; 42%). WEAA was standardized as the contents of anemarsaponin B (molecular weight; 902.794) donated by one author (S.Y. Kim) using a LC/MS instrument (Waters/ZQ 2000), consisting of a Waters M2695 liquid chromatographed alliance with a Waters 996 photodiode array absorbance monitor placed in series between the chromatograph and the quadrupole mass spectrometer. Liquid chromatograph separations were made by reversed phase chromatography column (100 mm×2.1 mm, 3 μm, Atlantis C18, Waters). The mobile phase consisted of water and acetonitrile (ACN) in the gradient mode as follows with a flow rate of 0.3 ml/min; 0–15 min, 10% ACN; 15–35 min, 90% ACN; 35–40 min, 10% CAN. The injection volume was 10 μl. All parameters of the APCI-MS system were optimized and selected based on generation of protonated molecular ions ([M+H]+) of the analysis of interest and production of characteristic fragment ions. The following instrumental parameters were used for APCI-MS detection of anemarsaponin B in the positive ion mode: capillary, 3.5 kV; cone, 40 V; hex 1, 20 V; aperture, 0 V; hex 2, 0 V; source temperature, 100°C; desolation temperature, 500°C; desolation gas, 600 l/h; cone gas, 40 l/h; low mass resolution, 15.0; high mass resolution, 15.0; ion energy, 0.5; multiplier, 650.

Transient Focal Cerebral Ischemia Animals were anesthetized in a chamber with a mixture of N₂O and O₂ (95/5) (Research Animal Care, U.S.A.). Body temperature was maintained at 37°C throughout surgery by a heating pad (Biomed S.L., Spain). ECG were monitored throughout the procedure (SurgiVet, Japan), consisting of a Waters M2695 liquid chromatographed alliance with a Waters 996 photodiode array absorbance monitor placed in series between the chromatograph and the quadrupole mass spectrometer. Liquid chromatograph separations were made by reversed phase chromatography column (100 mm×2.1 mm, 3 μm, Atlantis C18, Waters). The mobile phase consisted of water and acetonitrile (ACN) in the gradient mode as follows with a flow rate of 0.3 ml/min; 0–15 min, 10% ACN; 15–35 min, 90% ACN; 35–40 min, 10% CAN. The injection volume was 10 μl. All parameters of the APCI-MS system were optimized and selected based on generation of protonated molecular ions ([M+H]+) of the analysis of interest and production of characteristic fragment ions. The following instrumental parameters were used for APCI-MS detection of anemarsaponin B in the positive ion mode: capillary, 3.5 kV; cone, 40 V; hex 1, 20 V; aperture, 0 V; hex 2, 0 V; source temperature, 100°C; desolation temperature, 500°C; desolation gas, 600 l/h; cone gas, 40 l/h; low mass resolution, 15.0; high mass resolution, 15.0; ion energy, 0.5; multiplier, 650.

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Measurement of MPO Activity MPO activity in brain tissue was determined as an index of neutrophil accumulation, as previously described. In brief, brains were rapidly removed and cut into two 4-mm thick slices (3 to 7 mm and 7 to 11 mm from the frontal pole), and then separated into right and left hemispheres. Wet weights were immediately recorded. Each sample was homogenized in 20 wt/vol of 50 mM potassium phosphate buffer (pH 6.0) and centrifuged at 30000 g (30 min, 4°C). The supernatants were discarded, and the pellets suspended in 0.5% HTAB in 50 mM potassium phosphate buffer (pH 6.0) at an original tissue wet weight-to-volume ratio of 1:10. Samples were then immediately frozen on dry ice, and three freeze/thaw cycles were performed with sonication (10 s, 25°C) between cycles. After the final sonication, samples were incubated at 4°C for 20 min and centrifuged at 12500 g (15 min, 4°C). The supernatant so obtained (0.05 ml) was mixed with 0.95 ml of potassium phosphate buffer (pH 6.0) containing 0.167 mg/ml of O-dianisidine and 0.0005% hydrogen peroxide. Change of absorbance at 460 nm was measured by spectrophotometer equipped with a kinetic analysis capability (JASCO, Japan). One unit of MPO activity was defined as the amount that degraded 1 μmol of peroxide/min at 25°C. Tissue MPO activity was calculated using human MPO as standard.

MPO Immunohistochemistry Rats treated with vehicle or WEAA (200 mg/kg) were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and postfixed in the same fixative solution overnight and subsequently cryoprotected with 20% sucrose in 0.05 M phosphate-buffered saline (PBS, pH 7.4) for 48 h. Thereafter, 40-μm sections were prepared in the coronal plane using a cryostat.

Free floating sections were incubated for 24 h in PBS (4°C) containing anti-MPO antibody (1:500), 0.3% Triton X-100, 0.5 mg/ml of bovine serum albumin, and 1.5% normal horse serum. The sections were then incubated with biotinylated secondary antibody (1:200) for 90 min then with avidin-biotin-peroxidase complex (1:100) at room temperature for 1 h. The peroxidase was reacted with 0.02% 3,3′-diaminobenzidine and 0.01% H₂O₂ for about 5 min. After

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each incubation step, sections were washed three times with PBS.

**DPPH Radical Scavenging Assay** Free radical scavenging ability was determined using the stable DPPH dissolved in aqueous methanol. Various concentrations of 0.1 ml of sample solution were added to 1.9 ml of the DPPH radical solution (0.1 mM). After incubation at 25 °C for 30 min, absorbance was measured at 515 nm. The percentages of the DPPH radical remaining were calculated by comparing the absorbance between the sample and the control. A control solution consisted of 0.1 ml of aqueous methanol and 2.0 ml of DPPH radical solution. (−)-Epicatechin was used as positive control.

**Statistical Analysis** Values are expressed as means±S.E.M. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test for drug-treated groups versus control (vehicle-treated) group. Statistical significance was set at *p*<0.05. Student’s *t*-test was used to compare MPO activity with that of the control group.

**RESULTS**

**Standardization of WEAA** Quality control of WEAA was performed using anemarsaponin B isolated from dried *A. asphodeloides*. In this system, anemarsaponin B was detected at around 11.1 min (Fig. 1). The contents of anemarsaponin B in WEAA were 2.41±0.37% (*n*=5).

**Neuroprotective Effects of WEAA** MCA occlusion for 2 h followed by 22 h of reperfusion resulted in extensive and reproducible focal infarction and hemispheric swelling throughout the cortical and subcortical structures, as quantified 24 h after MCA occlusion. A large infarct was induced in 2-h ischemia/22-h reperfusion control animals as shown in Fig. 2 (total infarct volume was 346.35±14.44 mm³). In the

WEAA-treated group, the ischemic areas unstained by TTC staining were located mainly in the lateral part of the striatum and the surrounding cortical areas, which constitute the core of the infarct. The anterior and medial parts of the striatum and large areas of the cortex remained unaffected compared with the control group. Total infarct volumes in the ipsilateral hemisphere of ischemia-reperfusion rat were significantly reduced by WEAA treatment in a dose-dependent manner (Fig. 2). Total infarct volumes at doses of 50, 100, 200, and 400 mg/kg were reduced approximately by 8%, 29%, 48%, and 49%, respectively, versus the control group. Maximal protective effects of WEAA on ischemia were achieved at 200 and 400 mg/kg; no significant difference was observed between the 200 and 400 mg/kg treated groups.

**Fig. 1.** Typical Chromatograms for the Determination of Anemarsaponin B in Water Extract of *Anemarrhena asphodeloides* (WEAA)

A and B show representative chromatograms by HPLC for WEAA (A) and anemarsaponin B (B), respectively. Anemarsaponin B was detected at around 11.1 min in this system and confirmed by APCI-MS system as described in Materials and Methods. C shows the mass spectrum of anemarsaponin B (26-O-β-D-glucopyranosylfurost-20(22)-ene-3β, 26-diol-3-O-β-D-glucopyranosyl-(1→2)-β-D-galactopyranoside, C₄₅H₇₄O₁₈). The protonated molecule ([M+H]+) of anemarsaponin B was observed at 903.794 (m/z).

**Fig. 2.** Total Infarct Volume Was Measured 2 h after MCA Occlusion Followed by 22-h Reperfusion

Vehicle (*n*=10), WEAA (50, 100, 200, 400 mg/kg, *n*=8), and MCI-186 (10 mg/kg, *n*=8) were administered orally twice promptly prior to reperfusion and 2 h after reperfusion. Data represent means±S.E.M. The significance of differences between the total infarction volumes of the control, WEAA, and MCI-186 groups were determined by ANOVA followed by Duncan’s test [F(5, 44)=18.22, *p*=0.001]. **p*<0.01, ***p*<0.001 (vs. control group).
Moreover, treatment with MCI-186 (10 mg/kg, p.o.) reduced the infarct volume by 47%. Cerebral edema also occurs commonly during the acute phase of a large cerebral infarction, and we found that administration of WEAA after MCA occlusion significantly reduced brain edema in a dose-dependent manner. Brain edema in the control group was 13.99% at 24 h after ischemic injury. And, brain edema at doses of 50, 100, 200, and 400 mg/kg of WEAA was reduced by 2%, 14%, 37%, and 46%, respectively, compared with the control group.

**Therapeutic Time Window of WEAA**

WEAA (200 mg/kg, p.o.) was administered at 0.5 h and 2.5 h, 1 h and 3 h, 2 h and 4 h, and 3 h and 5 h after MCA occlusion, and significantly reduced infarct volumes by 53%, 52%, 48%, and 28%, respectively, compared with the control group (Fig. 3). Brain edema at 0.5 h and 2.5 h, 1 h and 3 h, 2 h and 4 h, and 3 h and 5 h after MCA occlusion was also reduced approximately by 50%, 42%, 38%, and 4%, respectively, versus control. Brain edema tended to be smaller when treatment started 3 h and 5 h after MCAO, but this was not statistically significant. These data indicate that the optimal therapeutic time for WEAA administration was 3 h in this ischemia-reperfusion rat model.

**Effects on MPO Activities**

Figure 4 illustrates MPO activity in the MCA territory in response to MCA occlusion. In the control group, MPO activity in the ipsilateral hemispheres (0.367±0.002 unit/g wet tissue) was significantly greater than that in the contralateral hemispheres. In rats given WEAA, mean MPO activity in the ipsilateral hemispheres (0.216±0.003 unit/g wet tissue) was significantly lower than that observed in the control group (p<0.001).

**MPO Immunohistochemistry Analysis**

MPO-positive cells were analyzed in the striatum (0.2 mm posterior to bregma) and cortex (3.6 mm posterior to bregma). In the sham group, almost no MPO-positive cells were identified in the striatal and cortical areas, and in the WEAA treatment group only minimal MPO-positive cells were observed in these areas 24 h after MCAO (Fig. 5). However, in marked contrast, the brains of control rats studied at 24 h contained dramatic accumulations of MPO-positive cells within pial and parenchymal blood vessels and within the cortical and subcortical parenchyma. MPO-positivity was most robust in areas of severe injury, and greater in the cortical regions than in the striatum (Fig. 5).

**DPPH Radical-Scavenging Activity**

The antioxidant activities of WEAA were assessed on the basis of the radical scavenging effect of the stable DPPH free radical. (-)-Epicatechin, which was used as reference standard, exerted the highest antioxidant activity and possessed significant free radical-scavenging properties (over 95% inhibition of DPPH), whereas WEAA was relatively inactive (only 16.4%
In animals treated with WEAA only minimal numbers of MPO-positive cells were observed at 24 h after MCAO, whereas in marked contrast the brains of control rats contained dramatic accumulations of MPO-positive cells both within pial and parenchymal blood vessels and within the striatum and cortex areas. In the sham group, almost no MPO-positive cells were observed in the striatum and cortex areas (data not shown). However, WEAA did not show significant free radical scavenging properties (only 16.4% inhibition of DPPH). Since inhibition of MPO activity plays a more critical role in injured brain tissue than DPPH radical-scavenging activity, it appears that the reduced brain infarct volume due to WEAA treatment is more likely due to reduced neutrophil infiltration. Analysis of the infarct volume and MPO activity data demonstrated a clear correlation between reduction of brain infarct volume and MPO activity of injured tissue rather than with DPPH radical-scavenging activity. However, it is difficult to exclude the involvement of radical scavenging effects because higher concentrations of WEAA inhibited DPPH radicals and protected neuronal cell death in rat hippocampal slices in culture deprived of oxygen and glucose (data not shown).

Using MCAO model, Chopp et al. 22) showed that treating rats with anti-Mac-1 antibodies also significantly reduced neutrophil accumulation in the ischemic cortex and subcortex. Furthermore, Matsuo et al. 9) reported reductions in the level of cortical and striatal neutrophils when ischemic rats were pretreated with an anti-neutrophil monoclonal antibody. Both studies showed a positive correlation between reduced neutrophil infiltration and the subsequently reduced ischemic injury. Recently, several reports suggest that PAF enhances presynaptic synthesis and release of glutamate in neuronal cultures and PAF-induced neurotoxicity involves the NMDA receptor/nitric oxide signal pathway. 23,24) It has also been shown that PAF receptor antagonists markedly prevent tissue injury and infarct volume in ischemic states. 25,26) Dong and Han 18) reported that anemarsaponin B isolated from A. asphodeloides inhibits platelet aggregation. As shown in the Materials and Methods section, anemarsaponin B is contained in high amounts in the extract of A. asphodeloides. If anemarsaponin B acts as a PAF antagonist and an active compound in WEAA, the neuroprotective effect of WEAA on ischemia-reperfusion-induced brain injury can be mediated by PAF inhibition. Unfortunately, more detailed examinations could not be undertaken because the amounts of anemarsaponin B available were insufficient to study the neuroprotective effect in rat MCAO model. Although the precise mechanism of the mode of action of WEAA and an active compound were not elucidated in the present study, it is apparent that the neuroprotective effect is mediated through a reduction of the number of invading neutrophils. Further studies on these issues are required.

In conclusion, the present study demonstrates that WEAA has a neuroprotective effect on ischemia-reperfusion-induced brain injury, and that this agent may act by reducing neutrophil accumulation in brain tissue. Although the action mechanism of WEAA has not been elucidated, it is believed that the anti-ischemic effect of WEAA is mediated via a reduction of neutrophil infiltration into brain tissue. These results suggest that WEAA should be viewed as a lead medicinal herb for the development of neuroprotective agents by providing neuroprotection in transient focal ischemic brain injury.

Acknowledgements This research was supported by the Kyung Hee University Research Fund in 2004 (KHU-20040333).

### Table 1. The Free Radical Scavenging Capacities of Water Extract of Anemarrhena asphodeloides

<table>
<thead>
<tr>
<th>Sample (mg/ml)</th>
<th>Absorbance (λ=515 nm)</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>1.51±0.06</td>
<td>—</td>
</tr>
<tr>
<td>(−)-Epicatechin 1</td>
<td>0.06±0.01</td>
<td>95.9±0.3</td>
</tr>
<tr>
<td>0.1</td>
<td>0.80±0.13</td>
<td>46.8±8.3</td>
</tr>
<tr>
<td>0.01</td>
<td>1.10±0.02</td>
<td>26.8±1.4</td>
</tr>
<tr>
<td>WEAA</td>
<td>1.26±0.02</td>
<td>16.4±1.3</td>
</tr>
<tr>
<td>0.1</td>
<td>1.32±0.08</td>
<td>12.4±5.4</td>
</tr>
<tr>
<td>0.01</td>
<td>1.38±0.04</td>
<td>8.5±2.6</td>
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(−)-Epicatechin was used as a positive control. The data shown are the means of triplicate measurements. (−)-Epicatechin showed strong activity by eliminating DPPH radicals at a concentration of 1 mg/ml.

DISCUSSION

In this study, we investigated whether treatment with WEAA can protect rat brain from injury induced by transient focal cerebral ischemia. We found that both ischemia-reperfusion-induced infarct volume and edema were reduced by WEAA in a dose-dependent manner, and that MPO activity was suppressed significantly in post-ischemic brain tissue.

*A. asphodeloides*, which is widely used in traditional Chinese medicine, is known to have anti-diabetic, anti-platelet aggregation, and diuretic activity, and various steroidal saponins have been isolated from *A. asphodeloides*. 11,12) Among these, timosaponin A-III and markogenin glycoside have been reported to inhibit ADP-, 5-HT-, and arachidonic acid-induced aggregation of human platelets. 12) Kaname et al. 17) also reported that timosaponin E1 and E2 inhibit platelet aggregation and delay activated partial thromboplastin time in a concentration-dependent manner. Moreover, anemarsaponin B was reported to have inhibitory activity against platelet aggregation induced by platelet activating factor (PAF) in vitro. 18) These results suggest that steroidal saponins from *A. asphodeloides* might be useful as novel antithrombotic therapeutic agents post-myocardial infarction. 13) However, no study has been undertaken on the neuroprotective effects of *A. asphodeloides* in ischemic brain damage.

WEAA was found to have a significant neuroprotective effect after double treatment in this rat reperfusion model, approaching 48% at 22 h similar to that of MCI-186 as positive control. MCI-186 has been reported to have inhibitory activity on ischemia-reperfusion in cultured brain slices and P AF-induced neurotoxicity involves the NMDA receptor/nitric oxide signal pathway. 23,24) It has also been shown that PAF receptor antagonists markedly prevent tissue injury and infarct volume in ischemic states. 25,26) Dong and Han 18) reported that anemarsaponin B isolated from *A. asphodeloides* inhibits platelet aggregation. As shown in the Materials and Methods section, anemarsaponin B is contained in high amounts in the extract of *A. asphodeloides*. If anemarsaponin B acts as a PAF antagonist and an active compound in WEAA, the neuroprotective effect of WEAA on ischemia-reperfusion-induced brain injury can be mediated by PAF inhibition. Unfortunately, more detailed examinations could not be undertaken because the amounts of anemarsaponin B available were insufficient to study the neuroprotective effect in rat MCAO model. Although the precise mechanism of the mode of action of WEAA and an active compound were not elucidated in the present study, it is apparent that the neuroprotective effect is mediated through a reduction of the number of invading neutrophils. Further studies on these issues are required.
REFERENCES AND NOTES


